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Dullard Promotes Degradation and Dephosphorylation of BMP Receptors and Is Required for Neural Induction

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Summary

Bone morphogenetic proteins (BMPs) regulate multiple biological processes, including cellular proliferation, adhesion, differentiation, and early development. In *Xenopus* development, inhibition of the BMP pathway is essential for neural induction. Here, we report that *dullard*, a gene involved in neural development, functions as a negative regulator of BMP signaling. We show that Dullard promotes the ubiquitin-mediated proteosomal degradation of BMP receptors (BMPRs). Dullard preferentially complexes with the BMP type II receptor (BMPRII) and partially colocalizes with the caveolin-1-positive compartment, suggesting that Dullard promotes BMPR degradation via the lipid raft-caveolar pathway. Dullard also associates with BMP type I receptors and represses the BMP-dependent phosphorylation of the BMP type I receptor. The phosphatase activity of Dullard is essential for the degradation of BMP receptors and neural induction in *Xenopus*. Together, these observations suggest that Dullard is an essential inhibitor of BMP receptor activation during *Xenopus* neuralization.

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Introduction

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the transforming growth factor-β (TGF-β) superfamily. Studies from transgenic and knockout mice revealed that BMP signaling is critical for vertebrate development (Zhao, 2003). Disruption of BMP signaling has been implicated in inherited cancers and various other diseases (Waite and Eng, 2003). Given these implications for human disease processes, it would be valuable to elucidate the mechanisms by which BMP signaling is regulated.

BMPs bind type I and type II cell-surface serine/threonine kinase receptors (BMPRI and BMPRII), thereby activating BMPRI, which, in turn, phosphorylates the C-terminal motif of BMP-Smads (Smad1, Smad5, and Smad8). These phosphorylated BMP-Smads then form a complex with Smad4 to activate the expression of BMP target genes in the nucleus. This pathway is regulated by multiple factors, including BMP antagonists, receptor-binding proteins, inhibitory Smads (Smad6 and Smad7), transcription factors, ubiquitin ligases, Smad phosphatases, and crosstalk with other signaling pathways (Chen et al., 2006; Derynck and Zhang, 2003; Massague and Chen, 2000).

In *Xenopus*, BMP signaling induces the ventral mesoderm and epidermis and negatively regulates neurogenesis (Reversade and De Robertis, 2005; Dosch et al., 1997; Wilson et al., 1997; Northrop et al., 1995; Schmidt et al., 1995). Secreted factors such as follistatin, noggin, and chordin are expressed in the organizer region of *Xenopus* embryos and induce early neural genes by directly neutralizing BMPs (Khokha et al., 2005; Ray and Wharton, 2001; Sasai, 2001).

We have shown previously that the *dullard* gene is essential for neural development in *Xenopus* (Satow et al., 2002). Here, we report that Dullard functions as an inhibitory factor for BMP signaling. Dullard specifically inhibits BMP signaling by promoting the ubiquitin-mediated proteosomal degradation of BMP receptors. Dullard functions as a phosphatase, and this activity is essential for BMP signal inhibition and the degradation of BMPRII. Furthermore, we provide evidence that Dullard also inhibits BMP signaling by dephosphorylating BMPRI. Our results suggest that Dullard plays a crucial role for neural induction during *Xenopus* embryogenesis by negatively regulating BMP signaling.

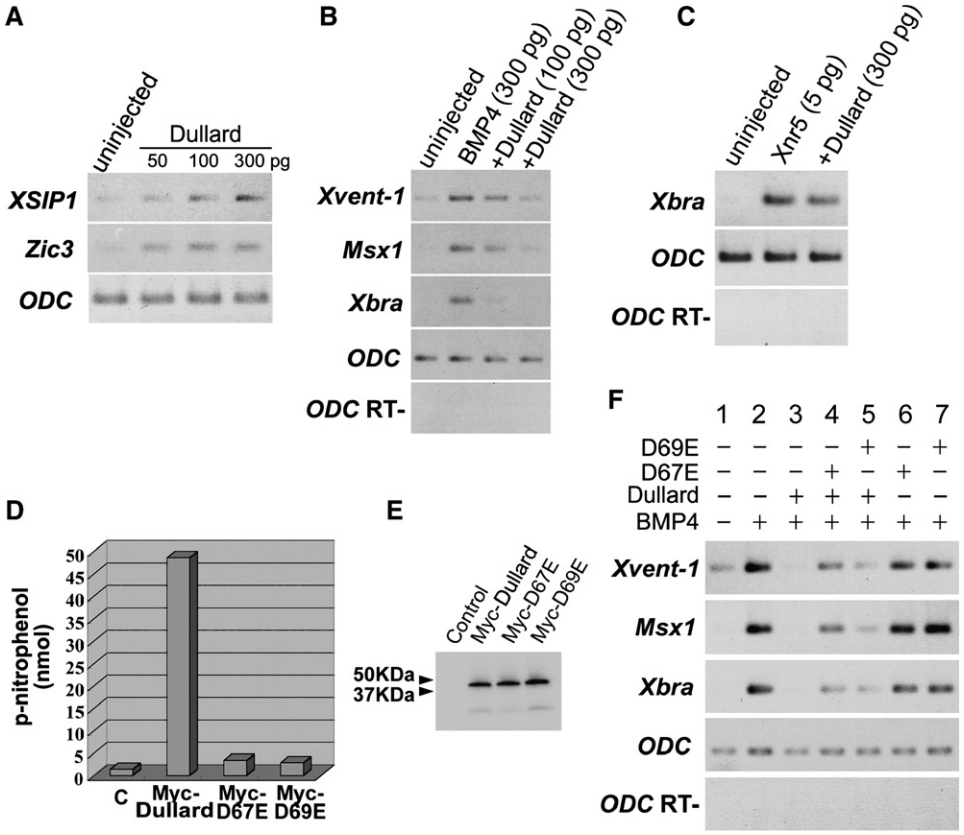

Figure 1. Dullard Is a Phosphatase that Suppresses BMP Signaling in *Xenopus* Ectoderm

(A) The increased expression of *XSIP1* and *Zic3* in *dullard* mRNA-injected animal caps. Uninjected or *dullard* mRNA (50, 100, or 300 pg)-injected animal caps were harvested at stage 13 for RNA isolation and RT-PCR.

(B) *dullard* suppressed the *BMP4*-induced expression of *Xvent-1*, *msx1*, and *Xbra* in a dose-dependent manner. *BMP4* and *dullard* mRNAs were injected as indicated. Animal caps were harvested at stage 10+, and the expression of *Xvent-1*, *msx1*, and *Xbra* was analyzed by RT-PCR.

(C) *Xbra* induced by *Xnr5* was not suppressed by coinjection with *dullard*. *Xnr5* (5 pg) and *dullard* (300 pg)-injected animal caps were harvested at stage 10+, and the expression of *Xbra* was analyzed by RT-PCR.

(D) Phosphatase activity of immunoprecipitated Dullard in vitro. Myc-tagged Dullard (wild-type) and the phosphatase-inactive mutants, D67E and D69E, were immunoprecipitated from embryos injected with the respective mRNAs. Control experiments were performed with uninjected embryos. Phosphatase activity was determined by measuring the amount of p-nitrophenol converted from the substrate, p-nitrophenol phosphate.

(E) Control blot of myc-Dullard proteins immunoprecipitated in (D).

(F) The expression of *Xbra*, *Xvent-1*, and *msx1* induced by *BMP4* was suppressed by coinjection with *dullard*, but not by D67E or D69E. Animal cap assays were performed. *BMP4* (300 pg), *dullard* (300 pg), D67E (1.5 ng), or D69E (1.5 ng) was injected as indicated. Animal caps were harvested at stage 10+, and RT-PCR was performed.

Results

Dullard Neuralizes *Xenopus* Explants by Antagonizing BMP Signaling

We showed previously that translational inhibition of *dullard* blocks normal neural development in *Xenopus* (Satow et al., 2002). To elucidate the mechanism underlying the contribution of Dullard to neural development, animal cap assays were performed and assessed the effect of *dullard* on the expression of early neural genes. As shown in Figure 1A, injection of *dullard* mRNA into animal caps upregulated the early neural genes *Zic3* and *XSIP1* in a dose-dependent manner. Since inactivation of the BMP pathway in ectodermal cells is essential for neural induction, we hypothesized that Dullard may induce neural development by antagonizing BMP signaling. To test this possibility, we coinjected *dullard* and *BMP4* mRNA into animal caps and analyzed the expression of the BMP target genes *Xvent-1*, *Msx-1*, and *Xbra*. As shown in Figure 1B, the expression of these genes was significantly suppressed by Dullard in a dose-dependent manner. We also examined the effect of *dullard* on activin-like signaling in animal caps and revealed that *Xbra* expression, induced by the overexpression of *Xnr5* (Takahashi et al., 2000), was unaffected by Dullard in coinjection experiments (Figure 1C). These results indicate that Dullard specifically antagonizes BMP signaling, but not activin-like signaling.

The Phosphatase Activity of Dullard Is Responsible for Its BMP-Repressing Ability

A BLAST domain search identified the catalytic phosphatase motif ψψψDXDX(T/V)ψψ (ψ: a hydrophobic amino acid residue) in Dullard. This motif is found in several serine/threonine phosphatases, including FCP1, a RNA polymerase II CTD phosphatase (Palancade et al.,

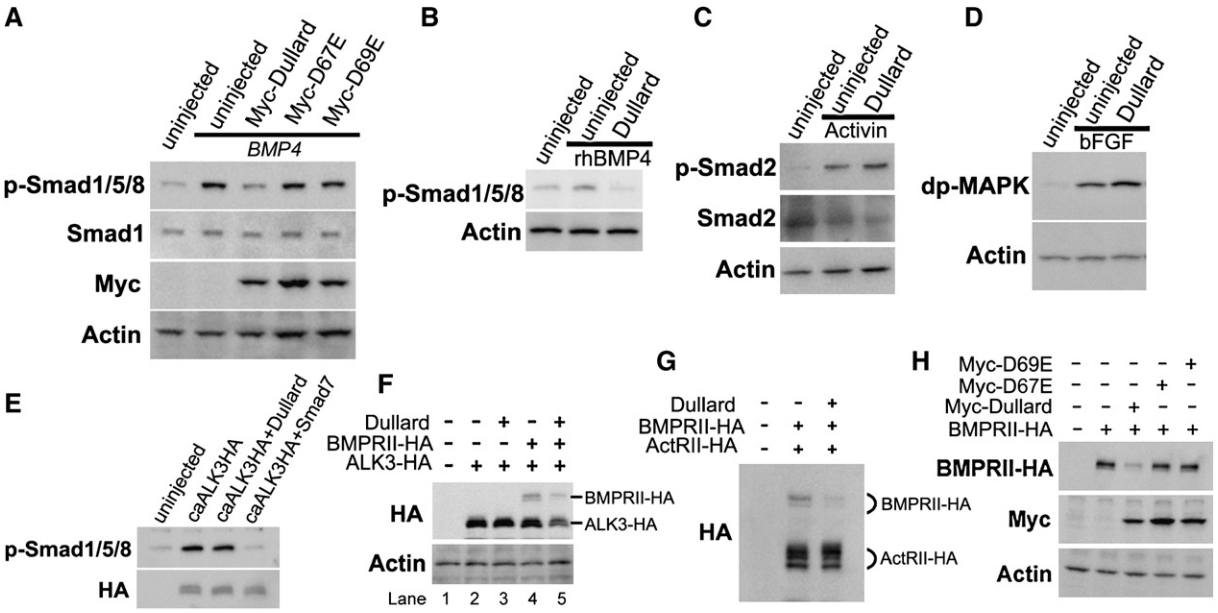

Figure 2. Dullard Promotes Degradation of BMP Receptors

(A) *BMP4*-induced phosphorylation of Smad1/5/8 was suppressed by coinjection with *dullard*, but not D67E or D69E. Animal caps injected with *BMP4* (300 pg), *dullard* (300 pg), D67E (300 pg), or D69E (300 pg) were dissected at stages 8.5/9, incubated until stage 10, and then analyzed by immunoblotting.

(B) Dullard inhibited recombinant BMP4-induced phosphorylation of Smad1/5/8. Uninjected or *dullard* (300 pg)-injected animal caps were dissected at stage 9, incubated with rhBMP4 (50 ng/ml) for 2 hr, and then harvested for immunoblotting.

(C and D) Dullard did not inhibit (C) activin-induced phosphorylation of Smad2 or (D) bFGF-induced phosphorylation of MAPK. The animal cap assay was performed as described in (B), but the caps were treated with activin (50 ng/ml) or with bFGF (150 ng/ml) for 2 hr.

(E) Dullard failed to suppress the phosphorylation of Smad1/5/8 induced by constitutively active ALK3. Constitutively active *ALK3* (caALK3-HA) (300 pg), *dullard* (300 pg), or *Smad7* (300 pg) was injected, and immunoblotting of phospho-Smad1/5/8 was performed as described in (B).

(F) The protein levels of BMP receptors were reduced by coexpression of Dullard. ALK3-HA (500 pg), BMPRII-HA (BRII-HA) (300 pg), and *dullard* (300 pg) were injected as indicated, and the receptors were detected with HA antibody.

(G) Dullard downregulated the protein level of BMPRII, but not ActRII. ActRII-HA (300 pg), BMPRII-HA (300 pg), or *dullard* (300 pg) was expressed as indicated.

(H) The protein level of BMPRII-HA was reduced by coexpression with Dullard, but not with D67E or D69E. BMPRII-HA (300 pg), myc-Dullard (300 pg), myc-D67E (300 pg), and myc-D69E mRNA (300 pg) were injected as indicated.

2001; Cho et al., 1999; Archambault et al., 1997, 1998). In order to test whether Dullard functions as a phosphatase, we made mutants of *dullard*, D67E and D69E, in which the first or second essential aspartate residue, respectively, within the catalytic motif was changed to a glutamate, as described previously in FCP1 (Hausmann and Shuman, 2002; Palancade et al., 2001).

In vitro phosphatase assays (Hausmann and Shuman, 2002) with immunoprecipitated myc-tagged Dullard using p-nitrophenyl phosphate as a substrate revealed that Dullard has significant phosphatase activity. In contrast, the D67E and D69E mutants retained only weak phosphatase activity compared to the wild-type control (Figures 1D and 1E). Animal cap assays also revealed that the phosphatase-inactive mutants had no BMP-antagonizing activity when coexpressed with *BMP4* (Figure 1F, lanes 6 and 7). Furthermore, coinjection of mutant mRNA with *dullard* reversed the expression of BMP-responsive genes that were suppressed by *dullard* alone (Figure 1F, lanes 4 and 5), indicating that D67E and D69E function in a dominant-negative manner. These results clearly show that the phosphatase activity of Dullard is responsible for its inhibitory effects on BMP signaling.

Dullard Blocks BMP Signaling by Promoting Degradation of BMP Receptors

To elucidate how Dullard represses BMP signaling, we examined the effect of *dullard* on BMP-dependent phosphorylation of Smad proteins with animal cap assays. Phosphorylation of Smad1/5/8 induced by injection of *BMP4* mRNA was reduced by coexpression with *dullard*. By comparison, the phosphatase-inactive mutants, D67E and D69E, did not suppress the BMP-dependent phosphorylation of Smad1/5/8 (Figure 2A). Dullard also inhibited the phosphorylation of Smad1/5/8 induced by treatment with the recombinant human BMP4 protein (rhBMP4) (Figure 2B). Dullard specifically suppressed the BMP-dependent activation of intracellular signaling, as evidenced by Dullard failing to suppress both the activin-induced phosphorylation of Smad2 (Figure 2C) and the bFGF-induced phosphorylation of MAPK (Figure 2D).

To understand mechanistically how Dullard inhibits BMP signaling, we next examined whether Dullard affects complex formation between the ligand and receptors or between type I and type II receptors. Dullard inhibited neither the binding between BMPRI, ALK3, and BMP4 (Figure S1; see the Supplemental Data available

with this article online), nor the BMP-dependent association of ALK3 and BMPRII (Figure S2). No binding was observed in control immunoprecipitation experiments for the binding of ALK4 and BMP4 (Figure S3), confirming the specific nature of the interaction between ALK3 and BMP4.

Dullard also blocked the phosphorylation of Smad1/5/8 induced by coinjection of BMPRII and ALK3 (Figure S4). Interestingly, when constitutively active ALK3 (caALK3) was expressed to activate BMP signaling (Wieser et al., 1995), Dullard failed to suppress the phosphorylation of Smad1/5/8, although an intracellular inhibitor of TGF-β family signaling, Smad7, completely inhibited the BMP-dependent phosphorylation of these Smad proteins (Figure 2E). These results suggested that Dullard inhibits the activation of BMPRIs, but not the activation of downstream signaling pathways. In these experiments, we noticed that the BMPR protein levels were reduced when coexpressed with Dullard (Figure S2), raising the possibility that Dullard downregulates BMPRII protein levels. This possibility was tested by Dullard overexpression, which caused a significant decrease in BMPRII (Figures 2F–2H), but not ALK3, protein levels (Figure 2F, lane 3). When ALK3 was coexpressed with BMPRII in the presence of Dullard, the protein levels of ALK3 decreased (Figure 2F, lane 5). These effects were BMPR specific, as Dullard expression did not change the activin-receptor level (Figure 2G). This result was confirmed by the finding that the phosphatase-inactive mutants, D67E and D69E, failed to decrease the protein level of BMPRII (Figure 2H). Together, these observations suggested that Dullard promotes degradation of BMPRs in a phosphatase activity-dependent manner.

Dullard Promotes Ubiquitin-Dependent Proteasomal Degradation of BMPRII and Dephosphorylation of ALK3

Dullard also inhibits BMP signaling in mammalian cells. A BMP-responsive mouse cell line, C2C12, was transfected with Flag-Dullard, treated with rhBMP4, and then immunostained with an anti-phospho-Smad1/5/8 antibody. While nontransfected cells showed distinct BMP-induced nuclear staining of phosphorylated Smads (data not shown; Figure 3B), cells expressing wild-type Dullard showed significantly weaker nuclear staining compared to surrounding nonexpressing cells (Figures 3A and 3B). This reduction in nuclear phospho-Smads was not seen, however, with the D67E mutant (Figures 3A and 3B). Western blotting analysis confirmed the Dullard-induced decrease in phosphorylation of BMP-Smads (Figure 3C). The overexpression of Dullard in mammalian cells further confirmed that Dullard reduced the protein level of BMPRII in a dose-dependent manner (Figure 3D). Interestingly, the degradation of BMPRII was reversed by treatment with MG132, a proteasomal inhibitor (Figure 3E). Dullard was also stabilized as well with MG132, suggesting that Dullard itself is degraded along with BMPRII (also see Figures 5B and 5C). Overexpression of Dullard markedly enhanced the ubiquitination of BMPRII (Figure 3F). The phosphatase-inactive mutant D67E showed reduced ubiquitination of the receptor, suggesting that the phosphatase activity of Dullard is important for this process. Together, these results indicate that Dullard promotes the ubiquitin-dependent proteasomal degradation of BMPRII.

We next analyzed the interaction between Dullard and the receptors by coimmunoprecipitation to elucidate the specificity of Dullard for receptor degradation. The assay was performed with cells harvested 24 hr posttransfection, as overexpression of Dullard significantly degrades BMP receptors in extended culture periods (data not shown), which causes uneven protein levels of the receptors in lysate. As shown in Figure 3G, BMPRII was coimmunoprecipitated with Dullard, but very low levels of ActRIIA were detected under the same conditions. These results indicate that Dullard specifically forms a complex with BMPRII, but not with the activin receptor. Similar experiments were performed to test the association of Dullard with the BMPRIs. As shown in Figure 3H, both BMPRII and ALK3 were coimmunoprecipitated with Dullard, although Dullard interacted more robustly with BMPRII than with ALK3. Testing of other BMPRIs, ALK2 and ALK6, revealed that these two receptors also interacted with Dullard (data not shown). Moreover, interaction between BMPRIs and Dullard was not dependent on the receptor kinase activity (data not shown). Furthermore, the interaction between Dullard and BMPRII was not dependent on the phosphatase activity of Dullard, as the phosphatase-inactive mutant Dullard still formed a complex with BMPRII (Figure 3I). These results indicate that Dullard preferentially forms a complex with BMPRII and promotes ubiquitination and degradation of BMPRs. Since Dullard is a serine/threonine phosphatase and binds to BMPRIs, it is possible that Dullard may also inhibit BMP signaling by dephosphorylating BMPRIs. To test this, Dullard was overexpressed, and the phosphorylation of ALK3 was examined. As expected, the BMP-dependent phosphorylation of ALK3 was significantly inhibited by coexpression with Dullard (Figures 3J–3L). To test whether Dullard directly dephosphorylates ALK3, we performed in vitro phosphatase assays by mixing immunoprecipitated Dullard and phosphorylated ALK3-HA immunoprecipitated from BMP-treated C2C12 cells. Wild-type, but not the phosphatase-inactive Dullard, facilitated the dephosphorylation of ALK3-HA in vitro (Figure 3M). These results demonstrated that, in addition to its role in BMPRII degradation, Dullard also inactivates BMP signaling by dephosphorylating BMPRIs.

Dullard Segregates BMPRII to Caveolae

Immunofluorescence analysis revealed a perinuclear, punctate expression of ectopically expressed Flag-Dullard in mammalian cells (Figure 4A). Dullard was localized in the cytoplasm, but not in the nucleus. By comparison, BMPRII-HA was localized diffusely at the cell surface. Coexpression of Flag-Dullard and BMPRII-HA markedly decreased the expression of BMPRII, with residual receptor colocalizing with Dullard in perinuclear puncta (Figure 4A). We further analyzed the dotted localization of transiently expressed Flag-Dullard in mammalian cells. As shown in Figure 4B, Dullard localized independently of an early endosome marker, EEA1, but partially colocalized with caveolin-1, a marker of the lipid raft-dependent caveolar pathway. These results suggested that Dullard expression might also segregate BMPRII to caveolin-rich membranes. To test this theory,

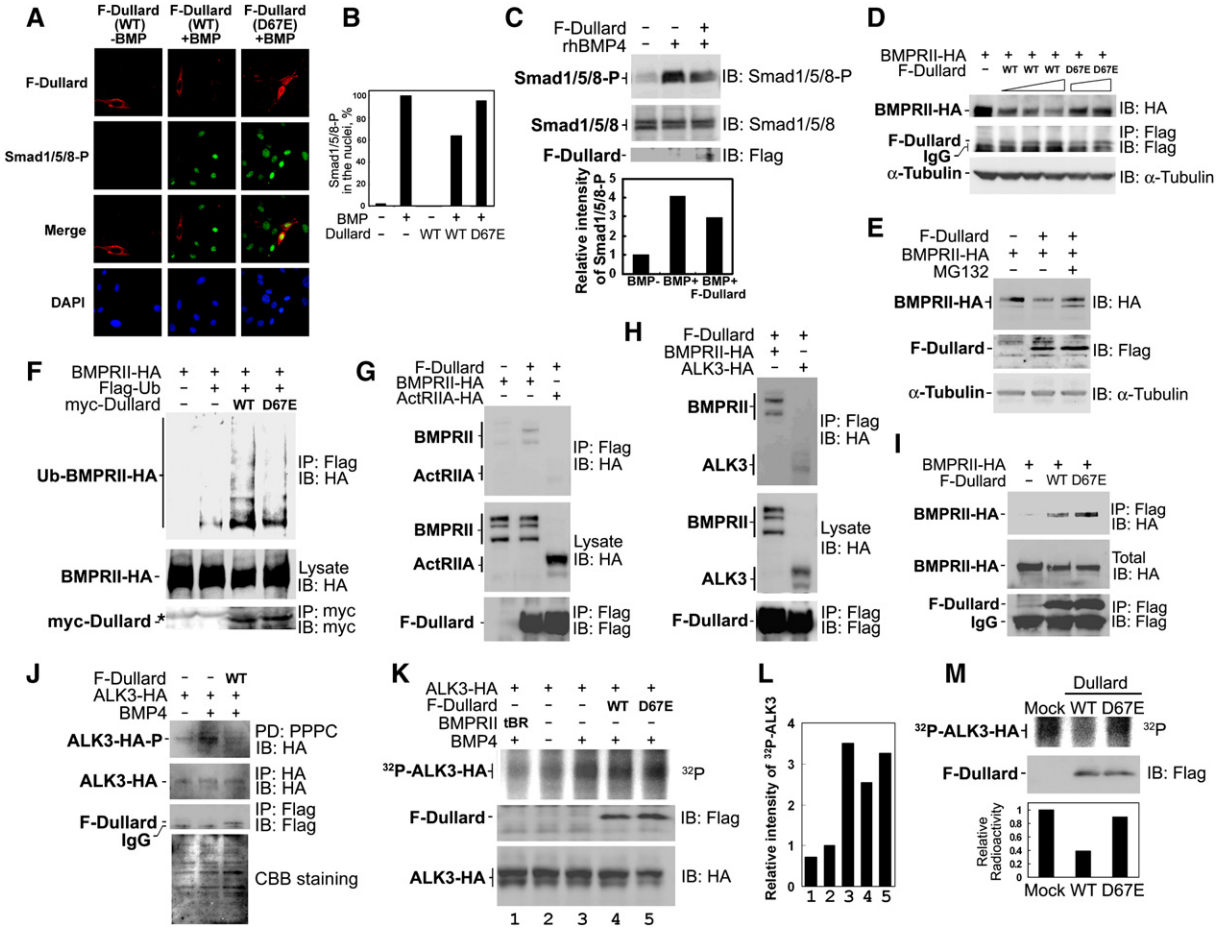

Figure 3. Dullard Promotes Ubiquitin-Dependent Proteasomal Degradation of BMPRII and Dephosphorylation of ALK3

(A) Dullard inhibited the nuclear staining of phospho-Smad1/5/8 in mammalian cells. C2C12 cells were transfected with Flag-Dullard, serum depleted for 24 hr, and then treated with rhBMP4 (100 ng/ml) for 30 min before immunostaining with phospho-Smad1/5/8 (green) and Flag (red) antibodies.

(B) Quantitation of phospho-Smad1/5/8-positive cells in (A).

(C) Dullard inhibited BMP-dependent phosphorylation of Smad1/5/8 in mammalian cells. A BMP-responsive human cell line, HepG2, was treated with rhBMP4 (50 ng/ml) for 30 min, and the lysate was immunoblotted with anti-phospho-Smad1/5/8 antibody.

(D) Dullard promoted the degradation of BMPRII in a dose-dependent manner. COS7 cells transfected with BMPRII-HA and a different dose of either wild-type or mutant Dullard were lysed 48 hr posttransfection, and BMPRII levels were detected by immunoblotting.

(E) Dullard promoted the degradation of BMPRII via the proteasomal pathway. BMPRII-HA and Flag-Dullard were used to transfect 293T cells as indicated. At 15 hr posttransfection, cells were treated with 15 μM MG132 for 15 hr, and the total lysate was prepared for immunoblotting. The amount of BMPRII DNA used in this experiment was the same for each lane. An immunoblot of α-tubulin is the loading control.

(F) Dullard promoted the ubiquitination of BMPRII-HA. Flag-ubiquitin, myc-Dullard, and BMPRII-HA were transfected as indicated. At 34 hr post-transfection, cells were treated with 15 μM MG132 for 8 hr, and immunoprecipitation was performed with Flag antibody. The ubiquitinated BMPRII was detected with HA antibody. The asterisk indicates nonspecific bands.

(G and H) Dullard preferentially formed a complex with BMPRII. Coimmunoprecipitation assays were performed with Flag antibody, and associated receptors were blotted with HA antibody.

(I) Dullard associated with BMPRII in a phosphatase activity-independent manner.

(J–L) Dullard inhibited BMP-dependent phosphorylation of the type I receptor. C2C12 cells were transfected, depleted of serum for 12 hr, and then treated with 100 ng/ml rhBMP4 for 15 min. (J) Phosphoproteins were purified on a PhosphoProtein Purification Column, and phosphorylated ALK3-HA was detected with HA antibody. (K) C2C12 cells were labeled with ^{32}P-phosphate before rhBMP4 treatment, and phosphorylation of immunopurified ALK3-HA with HA antibody was analyzed with a phosphoimager. (L) The phosphorylated ALK3 bands in (K) were quantitated.

(M) In vitro dephosphorylation of phosphorylated ALK3-HA by Dullard. Phosphorylated ALK3-HA beads prepared as in (K) were incubated at 30°C for 60 min with immunoprecipitated Flag-Dullard beads purified from transiently transfected COS7 cells. Dephosphorylation of ALK3-HA was analyzed as in (K) and (L).

cells were cotransfected with Flag-Dullard and BMPRII-HA, and the localization of BMPRII was examined. Expressed alone, BMPRII mainly localized at the cell surface (Figure 4C, Mock) and did not show apparent colocalization with caveolin-1, whereas in the presence of Dullard coexpression, BMPRII partially colocalized with caveolin-1, but not EEA1 (Figure 4C). These results suggest that Dullard recruited the receptor to the caveolin-dependent endocytic pathway to promote degradation of the receptor. In fact, the liganded endogenous BMP receptors were localized to both raft and non-raft compartments, as indicated by the sucrose-density fractionation of C2C12 cells affinity labeled with ^{125}I-BMP4 (Figure 5A). Ectopically expressed Dullard

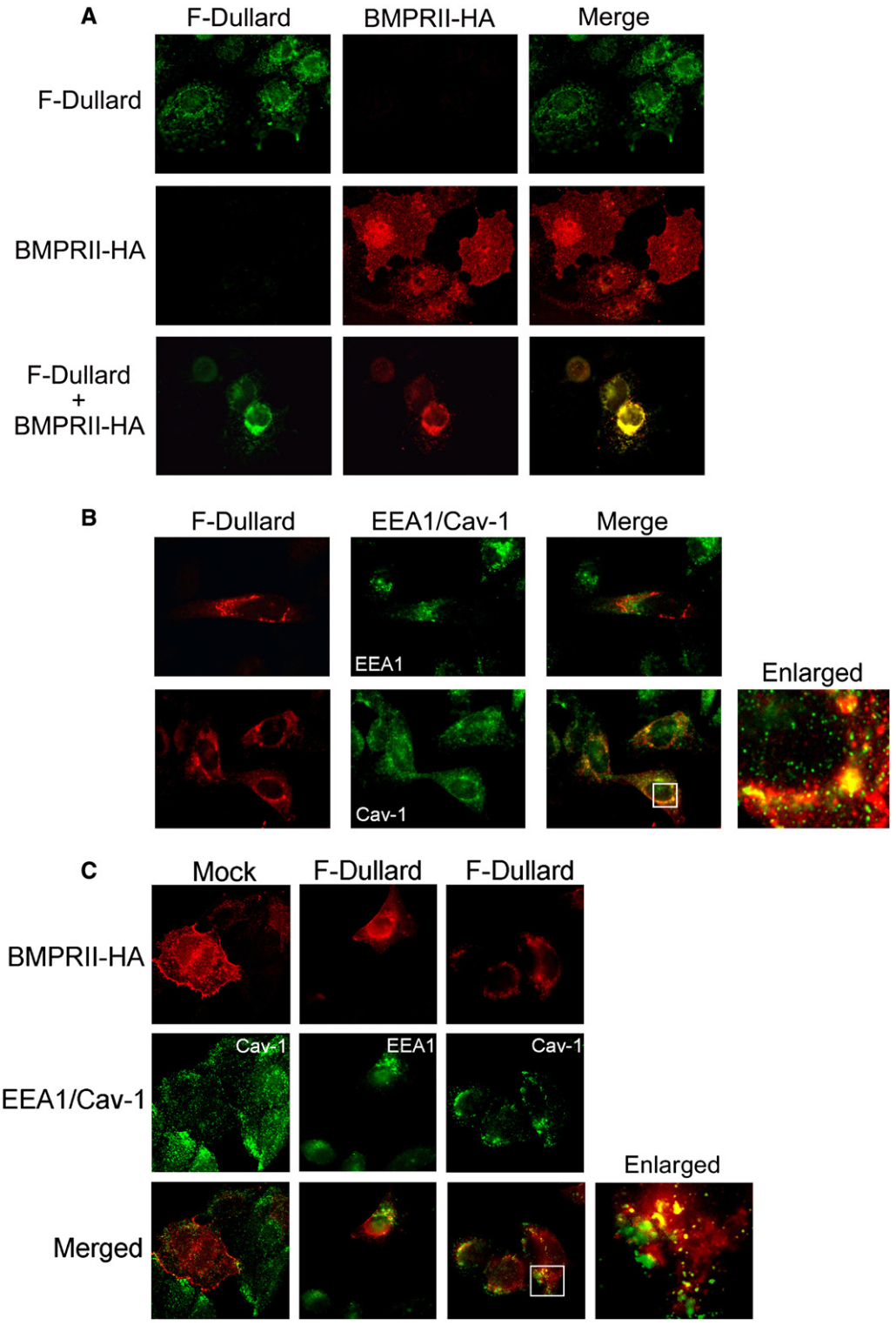

Figure 4. Dullard Recruited BMPRII to the Caveolin-1-Positive Compartment

(A) Subcellular localization of Dullard and BMPRII. Cells were transfected with Flag-Dullard (top lane), BMPRII-HA (middle lane), and both Flag-Dullard and BMPRII-HA (bottom lane). Immunostaining was performed with anti-Flag (left column) or anti-HA (middle column) antibodies.

(B) Dullard partially colocalized with caveolin-1, but not EEA1. HeLa cells were transfected with Flag-Dullard and immunostained with anti-Flag (left column) and either anti-EEA1 or anti-caveolin-1 (middle column) antibodies. Cells in the white square in the right bottom panel were further analyzed by confocal microscopy (enlarged).

(C) Dullard recruited BMPRII to the caveolin-1-positive compartment. HeLa cells transfected with both Flag-Dullard and BMPRII-HA were subjected to immunofluorescence. Cells were stained with anti-HA (top), anti-EEA1, or anti-caveolin-1 (middle) antibodies.

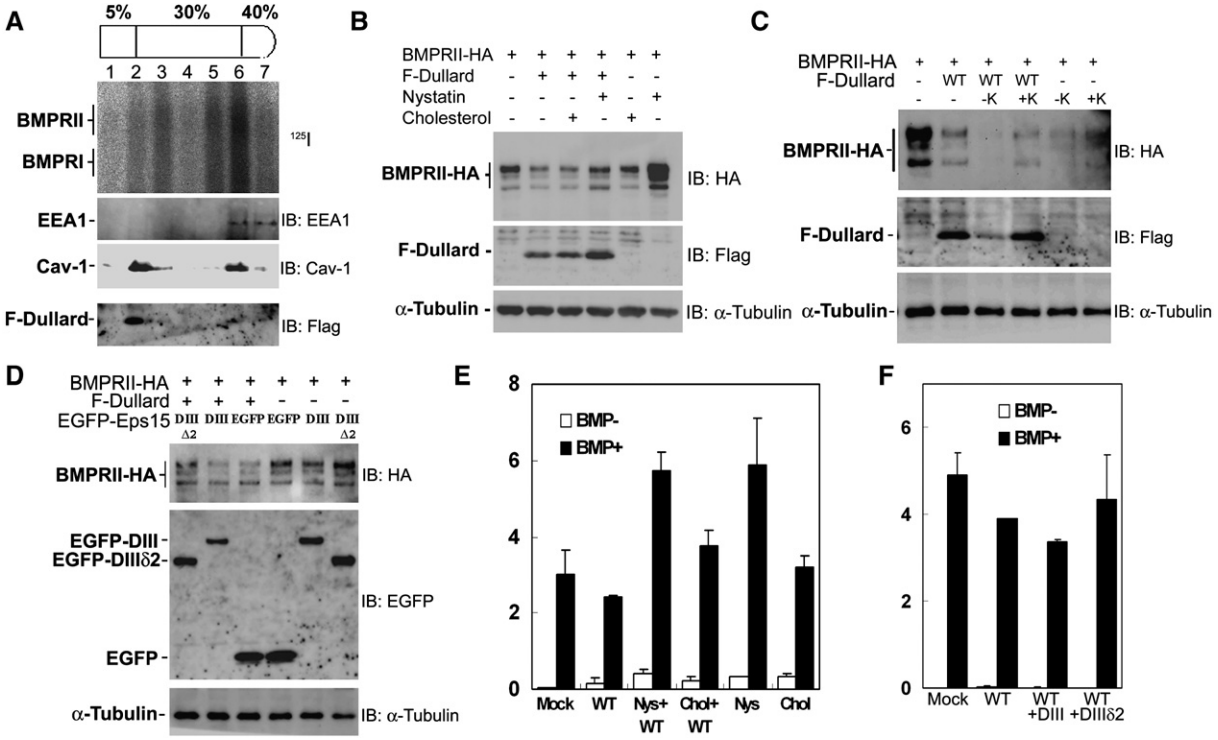

Figure 5. Dullard Promotes Degradation of BMPRII via a Caveolin-Dependent Pathway

(A) Liganded BMP receptors localized in both raft and non-raft compartments. C2C12 cells crosslinked with 125I-BMP4 were lysed and fractionated by sucrose gradient subcellular fractionation. The bottom panel shows an immunoblot with Flag antibody of independent fractionation of C2C12 cells transfected with Flag-Dullard.

(B) Nystatin inhibited BMPRII degradation induced by Dullard. COS7 cells transfected with indicated plasmids were treated with Nystatin (25 μg/ml) or cholesterol (25 μg/ml) for 14 hr.

(C) KCl deprivation enhanced the degradation of BMPRII by Dullard. COS7 cells transfected with indicated plasmids. The cells were cultured with or without 10 mM KCl for 5 hr 48 hr posttransfection.

(D) The dominant-negative Eps15 mutant did not inhibit BMPRII degradation by Dullard.

(E and F) Dullard-dependent inhibition of BMP signaling occurred via a caveolin-dependent pathway. Luciferase assays with a BMP-Smad reporter were performed with C2C12 cells as described in Experimental Procedures. The averaged data for normalized reporter activity are shown with standard errors derived from duplicate transfections.

also localized to raft compartments (Figure 5A, bottom panel). To further confirm this, inhibitors of the caveolar and clathrin endocytic pathways were included in our experimental system. Treatment with Nystatin, a cholesterol-depleting drug, known to abolish caveolae structure and its function, rescued the Dullard-induced degradation of BMPRII (Figure 5B), thereby implicating cholesterol-rich lipid raft compartments in the degradation. In contrast, KCl deprivation, which inhibits clathrin-dependent endocytosis, but not the caveolar pathway, rather promoted the degradation of BMPRII (Figure 5C), further supporting a role for a nonclathrin endocytic component. Furthermore, dominant-negative Eps15 (DIII), which also inhibits clathrin-dependent internalization by binding to the AP-2 adaptor (Benmerah et al., 1998) (but not the inactive variant, DIIIΔ2), failed to inhibit the Dullard-dependent BMPRII degradation (Figure 5D). The effects of these inhibitors on the degradation of BMPRII were also analyzed by luciferase assays. While treatment with Nystatin antagonized the Dullard-dependent inhibition of BMP signaling (Figure 5E), an Eps15 mutant, DIII, failed to rescue the inhibition by Dullard (Figure 5F). These data therefore support our model that Dullard promotes the degradation of BMPRII via a lipid raft/caveolin-dependent pathway.

Functional Knockdown of *dullard* Upregulates BMP Target Genes in Developing Embryos

To determine whether endogenous *dullard* also negatively regulates BMP signaling, animal cap assays were performed with D67E, D69E, and an antisense morpholino oligonucleotide for *dullard* (Dullard-Mo). D67E and D69E were shown in earlier experiments to act in a dominant-negative manner with regard to BMP signaling. Dullard-Mo was first reported previously (Satow et al., 2002), and as shown in Figure S5, this reagent efficiency blocked the translation. Figure 6A shows that injection of D67E or Dullard-Mo upregulated the phosphorylation of Smad1/5/8. RT-PCR analysis revealed that D67E, D69E, and Dullard-Mo also upregulated the expression of *Msx1*, a BMP target gene, and that this induced expression was suppressed by coinjection with dominant-negative BMPR (tBR) (Suzuki et al., 1994) (Figure 6B). Dullard-Mo also induced the expression of other BMP target genes, *Bambi* and *Xvent-1*, while control-Mo did not (Figure 6C). A second morpholino oligonucleotide for *dullard* (Dullard-Mo2), targeting the 5′ UTR sequence of *dullard*, also quite efficiently suppressed the expression of Dullard (Figure S5) and induced the expression of *Bambi* (Onichtchouk et al., 1999) and *Xvent-1* (Figure 6C). Luciferase assays with

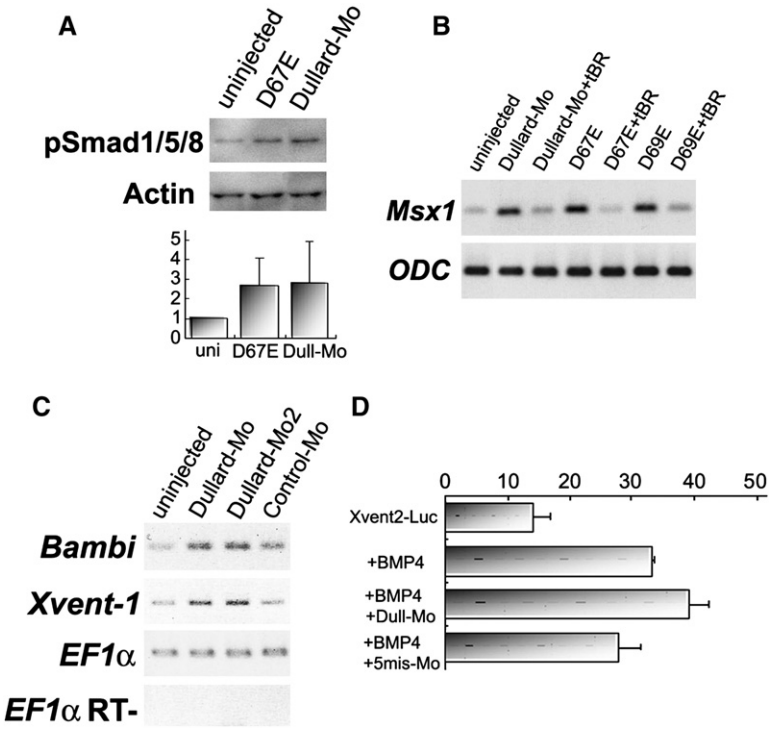

Figure 6. Inhibition of *dullard* Upregulates BMP Signaling

(A) Inhibition of *dullard* upregulated the phosphorylation of Smad1/5/8. Animal cap assays were done with Dullard-Mo (40 ng) or D67E (1.5 ng) mRNA-injected embryos. The relative intensity of the phospho-Smad1/5/8 immunoblot compared to actin was also measured. The averaged data for immunoblot are shown with standard errors derived from duplicate experiments.

(B) Inhibition of *dullard* induced the expression of *Msx1*. Dullard-Mo (40 ng), mRNAs of D67E (1.5 ng) and D69E (1.5 ng), or dominant-negative BMP receptor (tBR) (750 pg) was injected, and animal cap assays were performed to analyze the expression of *Msx1*.

(C) Knockdown of *dullard* upregulated the expression of *Bambi* and *Xvent-1*. The embryos injected with Dullard-Mo (40 ng), Dullard-Mo2 (20 ng), or control-Mo (40 ng) were subjected to animal cap assays, and the expression of *Bambi* and *Xvent-1* was measured.

(D) Luciferase assays were performed with the promoter of *Xvent2*. XVent2-Luc (50 pg), BMP4 (100 pg), Dullard-Mo (40 ng), and 5mis-Mo (40 ng) were injected into the animal pole region, and animal caps were dissected for luciferase activity. For an internal control, pRL-TK (50 pg) (Promega) was used. In each experiment, pGL-Basic (50 pg) (Promega) was also used, and the relative intensity to pGL-Basic is shown. The averaged data for normalized reporter activity are shown with standard errors derived from duplicate transfections.

the *Xvent2* promoter (Candia et al., 1997) revealed that BMP4-upregulated reporter activity was enhanced by coinjection with Dullard-Mo, but not by 5mis-Mo (Figure 6D). These results indicate that endogenous Dullard also inhibits BMP signaling and represses the expression of BMP response genes.

Dullard Contributes to the Expression of Early Neural Genes

We previously showed that translational inhibition of *dullard* blocked neural tube formation and inhibited head development (Satow et al., 2002). Here, we show that microinjection of Dullard-Mo or Dullard-Mo2, but not control-Mo or 5mis-Mo, significantly reduced the expression of the early neural marker genes *SoxD*, *XSIP1,* and *Zic3*, which are upregulated upon BMP inhibition (Figure 7). Furthermore, D67E and D69E exerted similar effects to Dullard-Mo and Dullard-Mo2 on the expression of these early neural markers. These results indicate that endogenous Dullard contributes to the expression of early neural genes by negatively regulating BMP signaling during development.

Discussion

The present study showed that Dullard functions as a regulator of BMP signaling that promotes ubiquitin-dependent proteasomal degradation of BMPRII and dephosphorylates BMPRIs. Dullard specifically inhibits BMP signaling and does not prevent other signaling pathways, such as activin/nodal or FGF signaling. As shown by binding studies, inhibition of BMP signaling is due to the specific targeting of BMPRs by Dullard.

Several proteins have been reported to promote the degradation of BMP-signaling components (Miyazono et al., 2005). To our knowledge, Dullard is the first protein shown to specifically promote the degradation of type II receptors of the TGF-β superfamily. Although we observed weaker interaction of Dullard with type I receptors, we failed to detect efficient degradation of ALK3 by Dullard, and expression of ALK3 was significantly downregulated only in the presence of BMPRII coexpression. These results therefore indicate that Dullard preferentially recognizes BMPRII and promotes degradation of BMPRII and of the complex formed with this receptor.

It has been shown that lipid raft/caveolin-dependent endocytosis is required for rapid TGF-β receptor turnover of the Smad7-Smurf2-bound receptor (Di Guglielmo et al., 2003). Recent studies suggest that lipid raft-enriched membrane domains might represent a general membrane location for ubiquitin ligases and related molecules (Le Roy and Wrana, 2005). The present study localized Dullard to cytoplasmic puncta, partially overlapping with caveolin, but not with the early endosome marker EEA1. Moreover, the observation that transient coexpression of Dullard promoted the ubiquitination of BMPRII, and that the proteasome inhibitor MG132 blocked its degradation, suggested that Dullard promotes ubiquitin-mediated proteasomal degradation of BMPRII by using a combination of as yet unidentified ubiquitin ligases that reside in the lipid raft/caveolin fraction.

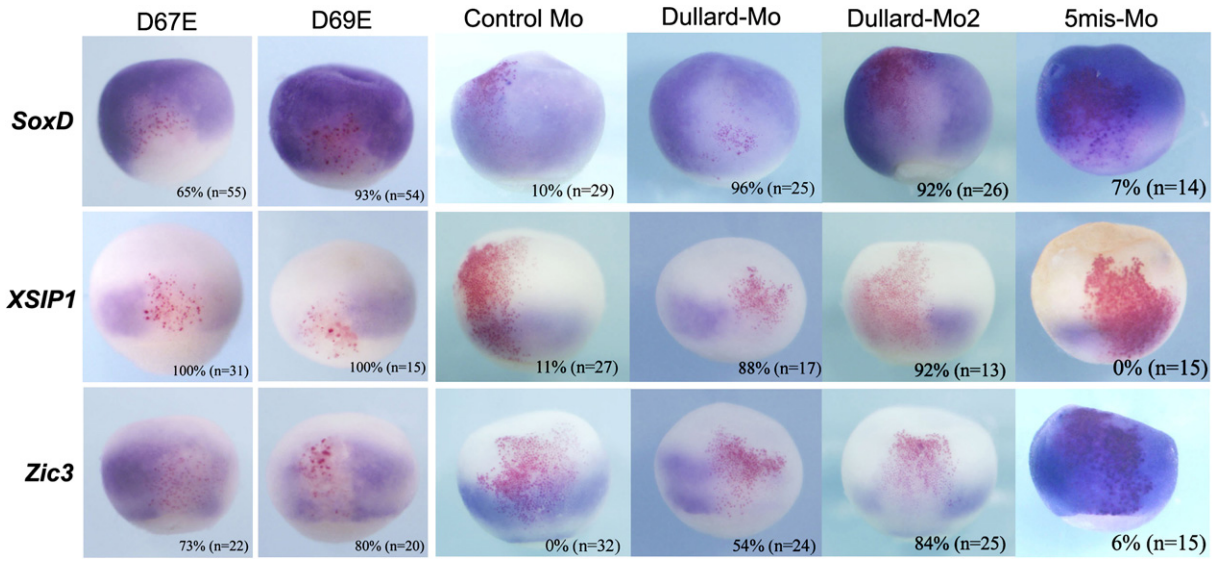

Figure 7. Inhibition of *dullard* Suppresses the Expression of Early Neural Genes

mRNA of D67E (1.5 ng) or D69E (1.5 ng), control-Mo (40 ng), Dullard-Mo (40 ng), Dullard-Mo2 (20 ng), or 5-mis Mo (40 ng) was microinjected into the marginal region with β-galactosidase mRNA as a tracer. After red-gal staining, whole-mount in situ hybridization for *SoxD*, *XSIP1*, and *Zic3* was performed. The percentage of embryos showing decreased expression and the total number of examined embryos are indicated in each figure. Figures for *XSIP1* and *Zic3* show the dorsal view.

The present study also found that Dullard functions as a phosphatase. As the substrates of the phosphatase, we identified BMP type I receptors. As shown in Figures 3J–3M, Dullard suppressed BMP-dependent phosphorylation of ALK3 by dephosphorylation. An intriguing finding is that the phosphatase activity of Dullard is also crucial for the degradation of BMPRII. Although we still do not know the mechanisms, it is plausible that Dullard may also recognize other unidentified substrates that regulate the ubiquitination and degradation of BMPRII. One possible explanation could be that Dullard may enhance the activity of the unidentified ubiquitin ligases by dephosphorylation of the ligases. This process could change interacting partners and modulate the ligase activity as described previously (Ichimura et al., 2005; Yang et al., 2006). Alternatively, Dullard inhibits BMP signaling by converting the constitutively phosphorylated BMPRII to a suitable dephosphorylated substrate for unknown ubiquitin ligases. There are several reports that describe specific degradation of unphosphorylated proteins by ubiquitin-dependent mechanisms (Tanaka et al., 2005; Katagiri et al., 2005; Coronella-Wood et al., 2004; Agarwal et al., 2003; Sato et al., 2003). To gain insight for the ubiquitin ligases involved in Dullard-dependent degradation of BMPRII, we tested a Smurf1-dependent mechanism. However, overexpression of a ligase-inactive mutant, Smurf1 (C710A), had no effect on the Dullard-dependent degradation of BMPRII. Moreover, coexpression of wild-type Smurf1 rather inhibited the Dullard-dependent degradation of the receptor (data not shown), suggesting that Dullard promotes the degradation of BMPRII in a Smurf1-independent manner. We speculate that Dullard functions as the phosphatase for BMPRIs and unknown components of the BMPR complexes to promote their dephosphorylation and subsequent signaling pathways such as ubiquitin-dependent degradation of the BMPRII.

We previously identified *dullard* as an essential gene for neural development (Satow et al., 2002). Here, phosphatase-inactive mutants, D67E and D69E, failed to promote degradation of BMPRII and acted as dominant-negative forms of Dullard. Dullard inhibition by these mutants or by Dullard-Mo upregulated the expression of BMP target genes and suppressed the expression of early neural genes. These results provide strong evidence that Dullard functions as a negative regulator of BMP signaling in vivo during *Xenopus* development.

Since *dullard* transcripts are maternally derived and are expressed throughout early embryogenesis until neurula stages (Satow et al., 2002), it is likely that Dullard acts by repressing the occasional phosphorylation of BMP type I receptors to bring about a very low level of activity of these receptors or to temporarily limit the signal duration, rather than to actively suppress BMP signaling in the early developmental stages. Alternatively, the activity of Dullard might be regulated by unknown posttranscriptional or posttranslational mechanisms to activate specifically in presumptive neural regions. In later stages, *dullard* expression is restricted to brain, eyes, neural region, branchial arches, and the pronephros (Satow et al., 2002). The expression pattern of *dullard* overlaps significantly with the expression pattern of *BMPRI* (*ALK3*) and partially overlaps with that of *BMPRII* (Shi et al., 2000; Frisch and Wright, 1998; Graff et al., 1994), suggesting that Dullard may also regulate BMP signaling in these regions late in development.

Very recently, one of the small C-terminal domain phosphatase family members, SCP2/Os4, was identified as a modulator of BMP-Smad phosphorylation (Knockaert et al., 2006). SCP2/Os4 is distantly related to Dullard, but it is exclusively localized in the nucleus. Dullard likely functions as a phosphatase for BMPRI in the cytoplasm, but not for BMP-Smads in the nucleus, since Dullard is only detected in the cytosol. Moreover, as shown in

Figure 2E, injection of *dullard* mRNA failed to inhibit the phosphorylation of Smad1/5/8 induced by caALK3. These results suggest that Dullard does not directly dephosphorylate Smad proteins. However, it is very interesting that similar phosphatases regulate the different signaling molecules of BMP signaling in the cytoplasm and nucleus. As these phosphorylation sites, the pSer-X-pSer motifs of both the GS domain of type I receptors and the C-terminal tail of Smads, show similarity and are both recognized by the same pSer binding motif, the Smad MH2 domain (Qin et al., 2001; Wu et al., 2001), the similar phosphatases could be responsible for the dephosphorylation of these motifs.

Experimental Procedures

Plasmid Construction

The catalytic domain mutants of *dullard*, D67E and D69E, were constructed by PCR amplification of pCS2-Dullard by using the T7 primer as the reverse primer and the following forward primers: 5′-CTAGTTCTAGAATTAGATGAGACCC-3′, for D67E, and 5′-CTAGTTCTAGATTTAGAAGAGACCC-3′, for D69E. The underlined sequence indicates the internal XbaI sites. The amplified products were digested with XbaI and cloned into the equivalent site in pCS2-Dullard. To construct myc-Dullard, myc-D67E, and myc-D69E, the ORF sequences of *dullard*, D67E, and D69E, respectively, were amplified by PCR and were then cloned into the StuI site of the pCS2-myc plasmid. pNRRX-XSmad7 was constructed by using the full-length cDNA clone of Smad7 obtained by our screening (Tanegashima et al., 2000). To construct pCS2-XBMPRII-HA and pCS2-ActRII-HA, the ORF sequence of *XBMPRII* (Frisch and Wright, 1998) and *ActRII* (AR1) (Hemmati-Brivanlou et al., 1992), respectively, was inserted into pCS2-HA. The constitutively active form of *ALK3* (pCS2-caALK3-HA) was constructed by inserting the ORF sequence of caALK3 and the HA tag sequence into pCS2. The wild-type of ALK3 plasmid, pCS2-ALK3-HA, was constructed by PCR-mediated modification of pCS2-caALK3-HA. The mammalian expression plasmids for wild-type and mutated Dullard were generated by PCR amplification of pCS2-myc-Dullard and were subcloned into the pcDNA3-Flag vector.

Embryos and Microinjection

Preparation of *Xenopus laevis* embryos and microinjection were performed as described previously (Satow et al., 2004). Staging of embryos was performed according to Niewkoop and Faber (1994). Capped mRNA was synthesized by in vitro transcription (mMESSAGE mMACHINE kit; Ambion). Plasmids encoding *BMP4* (Nishimatsu et al., 1992), *Xnr5* (Onuma et al., 2005), *dullard* (Satow et al., 2002), D67E, D69E, caALK3-HA, XBMPPRII-HA, ALK3-HA, ALK3-myc, and ActRII-HA were linearized with NotI and transcribed from the SP6 promoter. *XSmad7* was cut with SrfI and transcribed from the T7 promoter. BMP4-HA (Haramoto et al., 2004) was cut with Asp718 and transcribed from the SP6 promoter. In animal cap assays, mRNA was injected into the animal pole region of four-cell-stage embryos. Animal caps were dissected at stages 8.5–9 and were incubated in Steinberg's solution containing 0.1% BSA until harvesting.

RT-PCR

Total RNA isolation and RT-PCR were performed as described previously (Tanegashima et al., 2000). The primer pairs for *XSIP1* (Nitta et al., 2004), *Msx1* (Yamamoto et al., 2000), *Bambi* (Onichtchouk et al., 1999), and *ODC* (Chan et al., 2000) were described previously. The primer sequences of *Xvent-1* were: forward, 5′-AAGTATGC CAAGGAGATGCC-3′; reverse, 5′-AGCTTCTTCCGTTCAGATGC-3′. The primer pairs for *Zic3* and *Xbra* were as described in the *Xenopus* Molecular Marker Resource web site (http://www.cbrmed.ucalgary.ca/pvize/htm/WWW/Welcome.html). *ODC* and *EF1α* were used as loading controls. *ODC* and *EF1α* primers were also used in reverse transcriptase negative (RT−) reactions to confirm the absence of contaminating genomic DNA.

Antisense Morpholino Oligonucleotides

The antisense morpholino oligonucleotide for *dullard* (Dullard-Mo) was described previously (Satow et al., 2002). The second antisense morpholino oligonucleotide for *dullard* (Dullard-Mo2) was designed to target 5′-CGGTTACACTCCCATTCTGCTCTGG-3′, which is located in the 5′ UTR region of *dullard*. A GenBank search for the complement of the Dullard-Mo and Dullard-Mo2 sequences confirmed the absence of a significant homology sequence in *Xenopus*. A control Mo was provided by Gene Tools. 5mis-Mo carrying five mismatch nucleotides within the Dullard-Mo target sequence, 5′-CGAGGT GCCGTGGACTCCGCTTCAA-3′, was also used as a control.

Immunoprecipitation and Immunoblotting

Xenopus embryos, dissected animal caps, or cultured mammalian cells were lysed with a lysis buffer (1% Triton X-100, 250 mM NaCl, 50 mM HEPES [pH 7.0]) containing a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma) and were then centrifuged. Anti-phospho-Smad1/5/8 (Cell Signaling) was used to detect the C-terminally phosphorylated form of Smad1/5/8. Antibodies against Smad1/5/8, Smad1, Smad2, ALK3, caveolin-1, GFP, HA (Y11), and myc (9E10) were purchased from Santa Cruz. Antibodies against actin, α-tubulin, diphosphorylated-ERK1&2 (dp-MAPK), and Flag (M2) were purchased from Sigma. HA antibodies, 12CA5 and 3F10, were purchased from Roche. Other antibodies, including phospho-Smad2, BMPRII, and EEA1, were obtained from Cell Signaling, R&D, and BD, respectively. Anti-rabbit IgG-HRP (Sigma), anti-goat IgG-HRP (Sigma), and anti-mouse IgG-HRP (Cell Signaling) were used as secondary antibodies. Immunoblot was obtained with a LAS-1000pro image analyzer (Fuji Film), and the relative intensities of protein bands were quantitated with Image Gauge software (Fuji Film).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and five figures and are available at http://www.developmentalcell.com/cgi/content/full/11/6/763/DC1/.

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